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14. ABSTRACT

Digital gait analysis was used in rats to successfully assess the impact of sciatic nerve injury and to evaluate the timecourse of recovery of function. The first two groups of nerve repairs studied (nerve autograft and acellular nerve allografts) had similar outcomes in the rat. Functional recovery at 4 months post-injury repair was incomplete but did not differ significantly between nerve repair techniques. Cell seeding of acellular nerves was not successful using the described techniques. A microneedle vaccination array was employed to pierce the epineurium of the acellular nerve in hundreds of places and allow infiltration of cells. The outcomes of this technique are being analyzed. Nerves were harvested from Lewis rats for preparation of allografts for sciatic nerve repair, but the anatomic features of the nerve did not allow sufficient numbers of allografts to complete the proposed studies. Strain differences in nerve branching patterns rendered several of the nerves unsuitable for processing according to the vendor's protocol. Acellular nerve allografts from Sprague-Dawley rats were provided and will be used for the research studies. These nerves are incorporated into other rat strains without immunologic disturbances according to the vendor (AxoGen).

15. SUBJECT TERMS

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INTRODUCTION:

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INTRODUCTION:
The current research addresses repair of large gap peripheral nerve injuries. Clinically, nerve injuries greater than 3-5 cm have poor outcomes, regardless of repair techniques. One of factors limiting the re-growth of the axon across a large nerve gap may be the lack of trophic factors in the extracellular matrix of the interposed nerve graft. It is hypothesized that amniotic derived tissues possess trophic factors that support axonal re-growth and that incorporation of these tissues into an acellular nerve allograft will result in a nerve allograft with an enhanced potential to re-grow across a large nerve gap. This research will optimize cellular seeding of nerve allografts and functional assessment of that optimal construct in a rat sciatic nerve defect. Acellular nerve allografts with and without Amniotic Fluid Derived Stem Cells (AFS) will be used to repair large nerve gaps in rats (15 mm). The outcomes of these surgeries will be compared to those obtained with autograft nerve repairs that currently have the best outcomes for large-gap peripheral nerve repair. These techniques then will be employed in anon-human primate model (macaca fasciculata) of large-gap (6 cm) peripheral nerve injury and repair. Functional outcomes also will be assessed in this model. Finally, an intervention to prevent the degenerative changes that occur in neuromuscular junctions following delayed nerve injury/repair will be studied. If successful, the potential for the denervated muscle to regain function after nerve repair would be increased.

KEYWORDS:
Peripheral nerve injury, nerve allograft, amniotic derived stem cells, rats, macaca fasiculata, cell seeding of scaffolds

OVERALL PROJECT SUMMARY:
HYPOTHESES/OBJECTIVES
We hypothesize that acellular nerve allografts (ANA) can be seeded with amniotic fluid-derived stem cells (AFS) to promote and accelerate nerve regeneration. The presence of the AFS will provide support for the regenerating axons without the requirement of becoming Schwann cells. The specific aims to address this hypothesis are noted below:

SPECIFIC AIMS
Specific Aim 1: To demonstrate the ability to seed ANA with AFS using sub-atmospheric pressure (SAP) in vitro. Cell culture will be utilized to establish that the AFS cells remain on the allograft scaffold and that they do not differentiate into another cell type. Control cultures will employ ANA's with topically applied AFS but without SAP.

a. Follow-up experiments will examine Schwann cell migration in the presence of seeded allografts
b. Decellularization of species-specific mixed motor nerve tissue will be performed using decellularization and oxidation to improve the porosity of the allograft construct and enhance AFS cell seeding potential

Specific Aim 2: To establish the feasibility of using AFS seeded ANA's in large gap nerve repairs in vivo.

a. Rodent studies using ANA with/without AFS to repair large gap nerve defects
b. Enhancement of regenerative rate will be investigated
c. Motor end plate preservation studies to maintain muscle potential for re-innervation
d. Non-human primate studies in pre-clinical testing.

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Investigators: Initiating Principal Investigator – Thomas L. Smith, PhD
Partnering Principal Investigator – Zhongyu John Li, MD, PhD
Animal Use at this site: Animals will be used at this site

Progress over the past 12 months:

SOW Task 1 Specific Aim 1 (months 1-12):
In vitro studies to demonstrate the ability to seed Acellular nerve allografts (ANA) with Amniotic fluid derived stem cells and tissue (AFS) using subatmospheric pressure (SAP).

Task 1.1 (months 1-6) Cell seeding using SAP. Tests first will employ fibroblasts (NIH/T3T cells) and will examine the ability of the subatmospheric pressure seeding device (SAPSD) to improve penetration of the fibroblasts into the ANA. Secondarily, the magnitude and duration of exposure to SAP resulting in the greatest cell seeding density within the center of the ANA will be identified. Cell culture will be utilized to establish that the AFS cells remain on the allograft scaffold and that they do not differentiate into another cell type. Control cultures will employ ANA's with topically applied AFS but without SAP.

a. Decellularization of species specific mixed motor nerve tissue will be performed using decellularization and oxidation to improve the porosity of the allograft construct and enhance AFS cell seeding potential

Progress Task 1.1:

- Cell culture for Schwann cells has been established in the investigator's laboratory using explanted Schwann cells from donor rats.
    - Yields from explants are low, but that is expected. Improvements on the techniques are being employed to increase the yield of these cells.
    - This is a critical step because we will need to provide a cell culture environment that supports the cellularized nerve constructs.
    - A Schwannoma cell line also has been established so that pilot studies of cell seeding experiments can utilize adequate numbers of cells.

- Green Fluorescent Protein expressing fibroblasts (NIH/T3T cells) have been obtained and stocks of these cells are preserved in liquid nitrogen. These cells allow clear visualization of cell distributions within the experimental scaffolds.

- Material transfer agreements are in place and acellular nerve allografts for both humans and rats have been obtained from AxoGen.

- Material transfer agreements are in place and amniotic tissues have been obtained from NuTech (26-11-2013)

- Cell seeding experiments began in January 2014
    - Four series of cell seeding experiments have been performed using subatmospheric pressure (SAP) as well as static seeding. One million cells have been applied to scaffolds under SAP's of
        - - 40 cm H_2O
        - - 30 cm H_2O
        - - 20 cm H_2O
        - - 15 cm H_2O
    - Cell seeding of the ANA using SAP has not been adequate. The chambers providing SAP have been modified to maximize application of SAP to the acellular nerve scaffold.
- Sciatic nerves from 45 Lewis rats were harvested bilaterally, frozen in saline, and shipped to AxoGen for decellularization and processing. AxoGen could not obtain an adequate number of ANA from these donor nerves because the nerves from Lewis rats differ from those normally processed by AxoGen (from Sprague Dawley rats). AxoGen has provided us with ANA obtained

from Sprague Dawley rats and has documentation that these ANA can be implanted in Lewis rats.

Task 1.2 (months 6-12) Using the pressures established in 1.1, AFS will be seeded onto the ANA. Flow cytometry and cell markers then will be utilized to document that the AFS do not differentiate after being seeded onto the ANA. If the AFS undergo a phenotypic change after seeding on the ANA, the new phenotype will be identified and measures will be employed to prevent this differentiation.

- We are resolving the cell seeding issues noted above.

Task 1.3 (months 6-18) Cell culture will be employed to study the migration of Schwann cells onto the AFS seeded scaffold. Commercially available Schwann cells (from Schwannoma cell lines) will be co-cultured with the AFS seeded ANA's. Parallel studies of Schwann cell infiltration of non-AFS seeded ANA's also will be performed. The density of Schwann cells in the middle of the ANA's will be assessed histologically at three different time points after initiating co-culture of the Schwann cells. These time points will be at 12 hours, 24 hours, and 48 hours.

- Co-culture systems are being established
- Accellular nerve allografts for rats (Sprague Dawley) have been received from AxoGen

Task 1.4 (months 12-18, if necessary) If the cell seeding results of 1.3 are unacceptable (poor seeding of the ANA), nerves will be decellularized and oxidized according to the techniques of Whitlock et al. (2007). This technique results in a more porous allograft structure. If the oxidation of the nerve allograft tissue is too aggressive, the techniques can be modified by decreasing the concentration of and duration of exposure to peracetic acid during the oxidation phase of the tissue treatment.

Task 2 Specific Aim 2 (months 6-36): In vivo studies to establish the feasibility of using this construct in large gap nerve repairs.

Task 2.1 (months 6-18) – ANA with AFS for long gap nerve repairs will be studied using Lewis Rats as experimental subjects. A large gap nerve injury (1.5 cm) will be performed and the gap will be repaired immediately with an ANA construct alone (Group 1), an ANA construct with AFS cells (Group 2), or with an autograft (nerve segment is cut out, reversed, and sewn back in place)(Group 3). All surgeries will be performed using aseptic microsurgical technique. Outcomes of nerve injury/repair will be assessed at 1 month, 2 months, and 4 months post injury.

a. Outcomes – Outcomes assessed will include: Walking track analysis as an indicator of return of motor control. Walking track analysis will be performed at 1 month, 2 months, and 4 months post injury. Each animal will be compared to their preinjury walking track values. Use of this technique will permit use of the highly sensitive repeated measures analysis of variance for these animals. This technique will reveal even slight differences between groups. The number of animals required per group to achieve statistical power will be reduced using this experimental design.

Histologic analysis of nerve recovery at the end of 4 months. Axon counts on the post injury nerve segments will be performed according to the methods of Ma (2002, 2007). In addition, axon morphology will be assessed and compared between treatment groups.

Analysis of neuromuscular junction (NMJ) density. The number of neuromuscular junctions per mm2 of muscle tissue within the normal distribution of motor end plates will be determined and compared between groups. (Ma 2007, 2002)

Fate of AFS in ANA's following regeneration. Two approaches will be used: first, immuno-histochemistry will be employed to identify the AFS cells. In parallel, studies using green fluorescent protein

labeled AFS cells will be initiated. These will allow us to monitor the fate of the AFS cells after several weeks of implantation.

Muscle force generation will be assessed following the last walking track analysis to assess the degree of motor recovery. These studies will utilize techniques developed in this laboratory. (Stone 2007, 2011)

Progress Task 2.1:

Progress Q1

- A DigiGate video analysis system for quantifying gait in rats and performing walking track analysis has been purchased and delivered to our laboratories. The company CEO has provided on-site instruction in its use and we have begun training and assessing rat gait. The DigiGate computer is also connected to our institutional web server. This has allowed us to utilize and test the on-line assistance provided by the DigiGate company. (20-11-2013)
- Lewis rats, the strain identified for these studies have been obtained and we are learning techniques for training these animals to walk on the DigiGate. (05-12-2013)

Progress Q2

- Nerve autograft repairs of sciatic nerve injuries have been performed on the first six treadmill trained Lewis rats. These surgeries were uneventful and all animals have had their staples removed. The first animals to undergo nerve autograft repairs will be tested on the DigiGate device at 1 month post-surgery (first animals tested on 01-04-2014). Additional testing of these animals will be performed at two and four months post-surgery.
- Surgeries to create and repair sciatic nerve injuries will be performed in the next cohort of treadmill trained rats beginning 01-04-2014

Progress Q3

- Two groups of rats underwent surgical transection of the sciatic nerve on the left side with repair of the injured nerve using either a nerve autograft (Group 3; nerve segment obtained from the same rat) or a nerve allograft (Group 1; AxoGen supplied acellular human nerve of appropriate size).
- Rats were tested on the gait analysis device (DigiGate) before injury, and at 1 month, 2 months, and 4 months. In summary, several components of the rats' gait are significantly altered by sciatic nerve injury. Their gait parameters did not return to pre-injury values after 4 months. There were no remarkable differences between allograft and autograft nerve repair outcomes, which is, in itself, notable.
- Muscle function data also were collected and these results are still being analyzed.
- Gross muscle weights on the nerve injury side were significantly lower than on the intact contralateral side, suggesting muscle atrophy occurred following nerve injury. This atrophy was not reversed four months after nerve repair.

Progress Q4

- Histology is continuing to assess axon counts as well as neuromuscular junction density
- Tracking of AFS cells in-vivo is being pursued through nano-particle labeling of cells and use of a 9T MRI to image these cells

Task 2.2 (months 12-24) – Motor end plate preservation to increase functional recovery following denervation/reinnervation of the affected muscle will be studied in a separate cohort of rats. This group (n=10) will be subjected to nerve injury and repair using a 15 mm nerve defect and autologous nerve repair as in 2.1. A beta 2 agonist (fenoterol) will be administered via an osmotic minipump to the denervated gastrocnemius complex at a dose rate of 1.4 mg/kg/day in a total volume of 24 microliters. This drug and dosing regimen has been demonstrated to reduce and reverse muscle wasting in rats (Ryall 2003). It is hypothesized that it may

reverse the loss of NMJ surface area and number following denervation. This may allow greater recovery following reinnervation.

A control group of injured rats (n=10) treated with vehicle for the beta2 agonist only will also be studied. Muscle force generation and histology to examine neuromuscular junction density will be performed at 120 days.

Task 2.3 (months 18-36) – Large gap nerve repairs will be studied in nonhuman primates. The nerve reconstruction constructs utilized in study 2.1 [ANA construct alone (Group 1), an ANA construct with AFS cells (Group 2)] will be employed bilaterally in a randomized fashion (right arm v. left arm) to repair a large gap nerve defects (6 cm) in macaca fasciculata monkeys. Electrophysiologic testing as well as functional assessments (grasp and pinch ability) will be assessed longitudinally on a bimonthly basis (beginning 3 months post surgery) for 12 months following large nerve gap repair of the median nerve. At the end of 1 year, the animals will be euthanized. The median nerve from the elbow to the wrist crease will be removed bilaterally for histologic study and the muscle tissue of the thenar complex will be recovered bilaterally.

KEY RESEARCH ACCOMPLISHMENTS:
Nothing to report

CONCLUSION:
Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The ability to incorporate cells into nerve scaffold remains a research challenge. Current techniques are inadequate. The current research has tried two innovative approaches which have not been successful. This potential pitfall was recognized in the research plan and the project is pursuing methods to increase the permeability of the nerve epineurium. These include microneedle treatment as well as mild oxidation. Oxidation has been demonstrated to increase permeability and porosity in tendon, meniscus and bone. It is anticipated that the optimal oxidation paradigm can be developed.

PUBLICATIONS, ABTRACTS, AND PRESENTATIONS:
Nothing to report

INVENTIONS, PATNETS, AND LICENSES:
Nothing to report

REPORTABLE OUTCOMES:
Nothing to report

OTHER ACHIEVEMENTS
Nothing to report

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APPENDICIES

COLLABORATIVE AWARDS:

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